P/546-280

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:	Confirmation No.: 2921
Nozer M. Mehta, *et al.*	
Serial No.: 10/761,481	Group Art Unit: 1654
Filed: January 20, 2004	Examiner: Jeffrey E. Russel

For: IMPROVED ORAL DELIVERY OF PEPTIDES

VIA EFS-WEB
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

SECOND DECLARATION OF CO-INVENTOR
WILLIAM STERN UNDER 37 C.F.R. §1.132

I, William Stern, hereby declare that:

1. My background and relationship to the present patent application, and to its owner, are as stated in paragraphs 1-3 of the Declaration of Inventor William Stern Under 37 C.F.R. §1.132 executed by me on May 23, 2008 and previously filed ("Stern I").
2. This declaration is provided as a supplement to the Stern I declaration and in response to the issues raised by the Examiner regarding the Stern I declaration in ¶12 on pp. 10-12 of the Office Action mailed July 30, 2008.
3. Example 3 at pp.55-57 of the present specification compares the bioavailability of an analog of parathyroid hormone, i.e., PTH 1-34, with and without a C-terminal amide group. The present Office Action (at p. 11, lines 5-7) acknowledges that the two peptides tested, "differ only with respect to their amidation state", i.e., amidated vs. non-amidated. The Examiner objects, however, that the subject peptides are intraduodenally administered rather than orally administered as required by the claims and he argues that the declarant has not established that intraduodenal administration will accurately predict bioavailabilities of orally administered peptides.
4. In response, the intraduodenal administration of peptides is used to model the absorption of peptides from an acid resistant protective vehicle as described in claim 3 of this patent. Such a vehicle is designed to release its contents (i.e., the peptide,

citric acid and other components) in the intestine, the duodenum being the first site where such a vehicle could open. Although the acid resistant vehicle (and its contents) is orally administered, the contents cannot be released until the acid resistant protective vehicle is dissolved in the intestines. Thus, the results obtained by intraduodenal administration of peptides are predictive for the oral administration of peptides as described in the present patent application.

5. The Examiner states further at p. 11 of the present Office Action (see, e.g., the last paragraph on p. 11) that a showing by applicants that the differences in bioavailability shown for intraduodenal administration are predictive of differences in bioavailability for oral administration, would demonstrate unexpected results. Based on the explanation in the paragraph above, therefore, as to the reasons for intraduodenal instead of oral administration, as well as to why and how the results achieved with such intraduodenal administration are comparable with those which under different circumstances would be expected to be achieved with use of oral administration, I submit that the results achieved with PTH1-34 clearly demonstrate just such unexpected results as mentioned by the Examiner. However, the Examiner then goes on to state (see, e.g., p. 11 of the Office Action) that the unexpected results would only, in his view, be established for the single tested peptide, i.e., PTH(1-34)-NH_2 and that a single comparative result would not be deemed to be commensurate in scope with claims not limited to PTH(1-34)-NH_2
6. The Examiner's attention is therefore respectfully directed to Example 4 at pp. 57-59 of the present application. The subject example demonstrates the improved bioavailability attributable to the presence of a C-terminal amide on luteinizing hormone-releasing hormone. According to the Examiner at p. 11 of the Office Action, in Example 4 the test involves LHRH, "which is amidated at a location that is naturally amidated". In response I note for the Examiner's information that naturally occurring LHRH, i.e., as used in the Example, is not amidated. Thus, Example 4 provides a comparison between such naturally occurring LHRH, which is not amidated at all and LHRH that is amidated at the C-terminus, i.e., at a location where the peptide is not naturally amidated. It does not constitute a test with a peptide that is naturally amidated. I recognize, furthermore, that in Example 4 as in case of

Example 3 discussed above, the peptide was administered via inter-duodenal injection, instead of being orally administered. However, as I established in ¶4 above, such intraduodenal administration is an accurate predictor of the bioavailability of orally administered peptides. Example 4, then, provides additional significant evidence amounting to a showing of improved bioavailability for a second species of peptide (in addition to PTH(1-34)-NH_2) which is amidated at a location where such peptide is not naturally amidated.

7. Additional evidence to support the non-obviousness of the claims of the present application is provided by Example 1. As noted by the Examiner on p.10 of the Office Action, the example illustrates the effect of carboxy terminal amidation on the oral bioavailability of salmon calcitonin. That is, the example compares the bioavailability of an orally delivered glycine-extended salmon calcitonin with an amidated salmon calcitonin. I submit the results shown in, e.g., Table 5 on p. 52 of the application clearly establishes that the amidation of a peptide improves its bioavailability, i.e., in comparison to bioavailabilities of the corresponding peptide without such amidation. This is true whether or not the amidation is carried out at a location where the peptide is naturally amidated or not. In this regard the claim limitation, i.e., that the amidation takes place at a location where the peptide is not naturally amidated, was added simply to differentiate the peptides according to the present claims from peptides known in the art which are amidated naturally, i.e., at a location where they are 'naturally' amidated., such as those described in the Balschmidt et al. (USP 5,157,021), Habener (USP 5,120,712), Barbier et al. (USP 6,110,892) and Peri et al. (US 2004/0023882) references relied upon by the Examiner to reject some of the presently pending claims. It should also be noted that amidation of the peptide at the C-terminus confers carboxypeptidase Y resistance (Liebisch, et al. (1986) *Proc. Natl. Acad. Sci. USA*, 1936-1940). A copy of the Liebisch et al. reference is attached to this declaration for the Examiner's consideration. Thus, the significant difference between sCT and sCT-gly is not 1 amino acid but the presence of a terminal amide group on sCT.
8. The total effect of the evidence discussed above, therefore, is to conclusively demonstrate that: (1) the amidation of orally delivered peptides improves the

bioavailability of such orally delivered peptides and further that (2) it would not be obvious to one having an ordinary degree of skill in this art to amidate a peptide, particularly at a location that is not naturally amidated, in order to obtain a greater bioavailability of such peptide, in comparison to a corresponding peptide lacking such amidation.

9. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

7/27/09
Date

William Stern

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Neurobiology

Isolation and structure of a C-terminally amidated nonopioid peptide, amidorphin-(8–26), from bovine striatum: A major product of proenkephalin in brain but not in adrenal medulla

(brain neuropeptide/precursor/tissue-specific post-translational processing/post-translational modification)

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ABSTRACT **We have isolated and sequenced a C-terminally amidated peptide from bovine striatum. The peptide was purified to homogeneity by adsorption to XAD-2 resins and four different HPLC steps. Amino acid composition analysis and gas-phase sequence analysis revealed identity of this peptide with residues 8–26 of the proenkephalin-derived opioid peptide amidorphin, which we have recently isolated from bovine adrenal medulla. C-terminal amidation of amidorphin-(8–26) from bovine striatum was demonstrated by its stability to carboxypeptidase A digestion and full crossreactivity in a radioimmunoassay that required the C-terminal amide group as part of the recognition site. The nonopioid peptide amidorphin-(8–26), which lacks the N-terminal [Met]enkephalin sequence of amidorphin, is a major product of the opioid peptide precursor proenkephalin in the brain. In the adrenal medulla, however, where amidorphin occurs in remarkably high concentrations, amidorphin-(8–26) could not be detected. This is indicative of differential post-translational processing of proenkephalin in different tissues. In the brain, as opposed to the adrenal medulla, amidorphin is further processed at the typical cleavage signals of two basic residues, giving rise to the nonopioid peptide amidorphin-(8–26) and, possibly, to the opioid peptide [Met]enkephalin. Thus, proenkephalin in the brain might be considered as a precursor in which an opioid peptide is linked with a nonopioid peptide of possibly different biological function.**

Several neuropeptide precursor proteins have recently been discovered that contain sequences of more than one bioactive peptide. Striking examples of this type of precursor are the three opioid peptide precursors proopiomelanocortin, proenkephalin (= proenkephalin A), and prodynorphin (= proenkephalin B). They contain either multiple opioid peptide sequences (proenkephalin and prodynorphin) or one opioid peptide copy together with bioactive nonopioid peptides (proopiomelanocortin). The genes of these precursors have been isolated and sequenced (1–9). What peptide or peptides are produced and secreted by a cell, however, is a function not only of the particular mRNAs expressed in the cell but also of the production and distribution of specific enzymes involved in the post-translational proteolytic processing of the peptide precursors. These post-translational mechanisms are tissue specific and may, thus, enable the selective liberation of a particular set of bioactive peptides from a precursor containing more than one biologically active sequence.

Here we present the isolation and sequence of a C-terminally amidated peptide from bovine striatum. It consists of residues 8–26 of the proenkephalin-derived opioid peptide amidorphin, which we have recently isolated from bovine adrenal medulla (10). Thus, amidorphin-(8–26) lacks the N-terminal [Met]enkephalin copy of amidorphin. In contrast to amidorphin, the nonopioid peptide amidorphin-(8–26) does not occur in the adrenal medulla, but it is a major product of the opioid peptide precursor proenkephalin in the brain. This is an example of the tissue-specific processing of a precursor.

METHODS

Preparation and Properties of the Amidorphin Antiserum. To generate an antiserum to amidorphin, the synthetic peptide (custom synthesis by Peninsula Laboratories, San Carlos, CA) was linked to bovine thyroglobulin by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl and injected into a male New Zealand White rabbit, as described in detail (10). The resulting antiserum was used in the RIA; ^{125}I-labeled synthetic amidorphin served as a tracer. The RIA procedure is described in detail elsewhere (11). The highly specific antiserum requires the C-terminal amide group as a recognition site: on a molar basis, synthetic amidorphin-(20–26) crossreacted 100%, and synthetic amidorphin-(20–26)-OH (the nonamidated form) crossreacted 0.01%. Peptide F crossreacted 0.4%. All other peptides tested revealed a crossreactivity of less than 0.1%.

Isolation and Characterization of Amidorphin-(8–26). As starting material for the purification, 10 bovine brains were obtained from a local slaughterhouse and put on ice, within 15–30 min after death. The striata were dissected within 1 hr and boiled in 10 vol of 0.1 M HCl for 10 min. The tissue was homogenized in a blender and centrifuged for 60 min at 10,000 × *g*. The supernatant was treated with *n*-heptane to extract the lipids. The aqueous solution was acified with 20% trichloroacetic acid to a final concentration of 2% trichloroacetic acid and centrifuged at 15,000 × *g* for 60 min. The supernatant was then lyophilized to about 50% of the initial volume to remove the residual organic solvent and applied to a 300-ml column filled with XAD-2-adsorber resin (Serva, Heidelberg). After washing with water, the adsorbed substances were eluted with 600 ml of methanol. The methanol was removed at reduced pressure in a rotary evaporator. The resulting material was dissolved in 50 ml of 0.1% trifluoroacetic acid and adsorbed to a preparative Waters μBondapak alkyl phenyl reverse-phase HPLC column, equilibrated with 0.1% trifluoroacetic acid/5% (vol/vol) acetonitrile. The column was eluted with a linear gradient of acetonitrile (from 5% to 60% acetonitrile in 180 min). Fractions containing amidorphin immunoreactivity were lyophilized and further purified with four different analytical reverse-phase HPLC

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systems: (*i*) 50 mM NaH_2PO_4/5% (vol/vol) methanol, pH 7.0/acetonitrile, Altex ODS 5-μm C_{18} column; (*ii*) 0.1% trifluoroacetic acid/acetonitrile, Altex RPSC C-3 column; (*iii*) 50 mM NaH_2PO_4/5% methanol, pH 2.7 (1 ml of concentrated H_3PO_4 per liter)/acetonitrile, Altex ODS 3-μm C_{18} column; and (*iv*) 0.1% trifluoroacetic acid/acetonitrile, Altex ODS 3-μm C_{18} column. The flow rate was 1.1 ml/min in *i–iii* and 1.0 ml/min in *iv*, and 0.5-ml fractions were collected.

Gas-phase sequence analysis was performed as described (10, 12). For amino acid composition analysis, peptides were hydrolyzed in 6 M HCl containing 1% phenol. Amino acids were then derivatized with phenylisothiocyanate and analyzed by reverse-phase HPLC (phenylisothiocyanate amino acid analysis, ref. 13).

For carboxypeptidase A digestion, a mixture of 200 pmol of purified amidorphin-(8–26) and 100 pmol of synthetic dynorphin A-(1–8) was dissolved in 1500 μl of 0.02 M Tris·HCl/0.2 M NaCl/0.1% Triton X-100, pH 7.5. A suspension of 10 μl of carboxypeptidase A (20 mg/ml) (Sigma type I) containing 200 μg of enzyme was dissolved in 1 ml of 10% LiCl. Then 5 μl of the freshly prepared solution containing 1 μg of enzyme was added to the peptide mixture and the sample was incubated at 37°C. Six 20-μl aliquots were withdrawn, after 1, 5, 10, 60, 120, and 300 min. To terminate the enzyme reaction, each sample was incubated for 10 min at 96°C. Finally, the samples were each assayed in quintuplicate by RIA for immunoreactive amidorphin and dynorphin A-(1–8). Zero-time samples were withdrawn before addition of enzyme to determine the amount of peptide initially present in the sample. Dynorphin A-(1–8) was used as a control substance, because for this peptide a RIA specific for the C terminus of dynorphin A-(1–8) was available (14). Separate samples of the peptide mixture with or without enzyme solution after inactivation at 96°C for 10 min were incubated for 300 min and assayed for immunoreactive peptides, to exclude the possibility of nonspecific degradation of the peptide during incubation.

Analysis of Amidorphin-Related Peptides in Bovine Tissue. Bovine brains, pituitaries, and adrenals were obtained from a local slaughterhouse and placed on ice within 30 min after death. The brains were dissected within 1 hr into the various areas, the pituitaries were separated into anterior and posterior lobes (neural lobe with adhering intermediate lobe), and the adrenal cortex was separated from the adrenal medulla. Tissues were extracted as described above. This extraction procedure, yielding a recovery of more than 85% for ^{125}I-labeled amidorphin, is superior to other extraction procedures, such as acid acetone in the presence of enzyme inhibitors or 2 M acetic acid (11). After centrifugation, 2-g equivalents of pooled tissues from eight animals were used for gel filtration on a Sephadex G50 superfine column (1.6 × 90 cm). The fractions containing immunoreactive amidorphin were collected and, after lyophilization, treated with 3% (wt/vol) H_2O_2 for 30 min at 56°C, to oxidize the methionine residues of immunoreactive amidorphin. Subsequently, immunoreactive amidorphin was applied to an ODS C_{18} 5-μm reverse-phase HPLC column (Altex, Berkeley, CA). It was eluted with 50 mM NaH_2PO_4/5% methanol, pH 7.0, and a linear gradient of acetonitrile from 0% to 30% within 120 min, with a flow rate of 1.1 ml/min. The fraction size was 2.0 ml. Aliquots of each fraction were lyophilized and analyzed for their content of immunoreactive amidorphin.

RESULTS

Isolation and Structure of Amidorphin-(8–26). Amidorphin-(8–26) was purified to homogeneity from bovine striatum extracts, using the chromatographic steps described. Two separate purifications of 10 bovine striata each (about 220 g) were performed, yielding an average of 3 nmol of pure amidorphin-(8–26). Fig. 1 shows the absorbance and immunoreactive profiles of the analytical HPLC step *iii*. Both of the major UV-absorbing peaks were detected by the amidorphin antiserum, which was highly specific for the amidated C terminus of the peptide. These two peaks (A and B) were desalted in a final HPLC purification step (step *iv*).

Amino acid composition analysis and gas-phase sequence analysis of peak A and peak B revealed unambiguously that the primary sequences of the peptides in both peaks were identical to the sequence 8–26 of amidorphin and correspond to the sequence 112–129 of bovine proenkephalin. This is shown for peak A in Tables 1 and 2.

The C-terminal amide group of peaks A and B was confirmed by carboxypeptidase A digestion experiments and by the immunological characteristics of these peptides. Endogenous peaks A and B from bovine striatum were completely stable for at least 300 min, whereas dynorphin A-(1–8), which was added together with peaks A and B to the enzyme mixture as a nonamidated control peptide, was completely digested by the enzyme within 5 min (see Fig. 2 for peak A). Moreover, peaks A and B exhibited full crossreactivity with the amidorphin antiserum that required the C-terminal amide residue as part of the recognition site.

Oxidation of peak A resulted in a shorter retention time on HPLC; this time was then identical to the retention time of peak B. Oxidation of peak B, however, did not alter the retention time of this peptide. Thus, we conclude that peak B represents the oxidized form of peak A, and that the full sequence of both peptides is Met-Asp-Glu-Leu-Tyr-Pro-Leu-Glu-Val-Glu-Glu-Glu-Ala-Asn-Gly-Gly-Gly-Val-Leu-NH_2. Since amidorphin-(8–26) contains a methionine residue, partial oxidation of this amino acid to methionine sulfoxide during the purification procedure is conceivable.

Regional Distribution of Amidorphin-Related Peptides. Amidorphin-related peptides were investigated in bovine adrenal medulla, posterior pituitary, and various brain areas by the combined use of a RIA specific for the C terminus of amidorphin, gel filtration, and HPLC. Amidorphin-related peptides were prepurified by gel filtration, in which they eluted in each case as a single immunoreactive peak (11). Prior to HPLC, immunoreactive peaks were oxidized, as were peptides used for calibration of the HPLC columns. Fig.

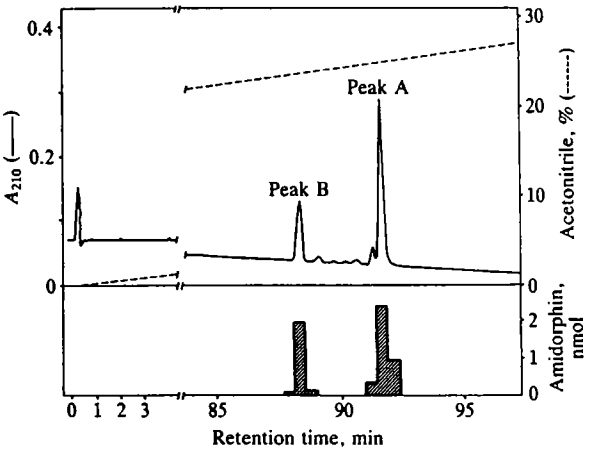

FIG. 1. Final step (step *iii*) of HPLC purification of amidorphin-(8–26) from bovine striatum. The figure shows the UV detection and the amidorphin-immunoreactivity profile (on a per fraction basis), determined with an antiserum that is highly specific for the C terminus of amidorphin and requires the C-terminal amide group in the recognition site. The peptide was prepurified by adsorbance to XAD-2 and HPLC as described in *Methods*. Prior to analysis of amino acid composition, peak A and peak B were desalted by HPLC.

Table 1. Gas-phase amino acid sequence analysis of amidorphin-(8–26) from bovine striatum

Cycle no.	PhNCS amino acid	Yield, pmol	Cycle no.	PhNCS amino acid	Yield, pmol
1	Met	269	11	Glu	148
2	Asp	62	12	Glu	149
3	Glu	201	13	Ala	92
4	Leu	218	14	Asn	73
5	Tyr	224	15	Gly	74
6	Pro	128	16	Gly	94
7	Leu	146	17	Glu	57
8	Glu	148	18	Val	40
9	Val	132	19	Leu	12
10	Glu	147	20	—	—

A 330-pmol portion of the purified peptide (peak A, Fig. 1) (determined by amino acid analysis) was loaded on an Applied Biosystem 470 A protein sequencer. Trifluoroacetic acid was used for conversion of anilinothiazolinone amino acids, and HPLC on an IBM-cyanopropyl column was used for identification of phenylthiohydantoin derivatives of amino acids.

3 shows the differential HPLC profiles of immunoreactive amidorphin in various tissues. In the adrenal medulla, the total amidorphin immunoreactivity was found to elute in the position of synthetic amidorphin, whereas amidorphin-(8–26) could not be detected. In the posterior pituitary, hypothalamus, midbrain, brainstem, and striatum, however, amidorphin-(8–26) was the predominant (in the striatum even the exclusive) form of immunoreactive amidorphin. [The identities of peaks 1 and 2 with amidorphin-(8–26) and amidorphin, respectively, were also demonstrated by use of a second HPLC system (data not shown).] Table 3 compares the concentrations of amidorphin with those of amidorphin-(8–26), and shows a differential distribution of these two peptides in different tissues. Amidorphin-(8–26) could be detected in particularly high concentrations in the posterior pituitary, hypothalamus, and striatum.

To exclude the possibility of an artifactual formation of amidorphin-(8–26) from amidorphin as a consequence of

Table 2. Amino acid composition of endogenous amidorphin-(8–26) from bovine striatum

Amino acid	Residues per molecule	Nearest integer	Expected integer	Difference
Asx	1.76	2	2	None
Glx	6.05	6	6	None
Gly	2.17	2	2	None
Ala	1.05	1	1	None
Pro	1.02	1	1	None
Tyr	0.99	1	1	None
Val	1.79	2	2	None
Met	0.76	1	1	None
Leu	2.87	3	3	None
His	0.00	0	0	None
Ser	0.19	0	0	None
Arg	0.22	0	0	None
Thr	0.09	0	0	None
Cys	0.00	0	0	None
Ile	0.00	0	0	None
Phe	0.02	0	0	None
Lys	0.00	0	0	None

This table presents the average result of four separate hydrolyses of amidorphin-(8–26) (peak A, Fig. 1). Duplicates of each hydrolysate were analyzed for amino acid composition. Values are not corrected for hydrolysis losses. Cys was determined as cysteic acid.

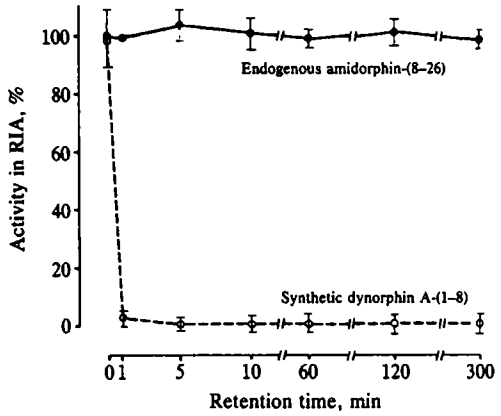

FIG. 2. Effect of carboxypeptidase A digestion on endogenous amidorphin-(8–26) from bovine striatum (peak A, see Fig. 1) and synthetic dynorphin A-(1–8). Endogenous amidorphin-(8–26) was stable for at least 5 hr, whereas dynorphin A-(1–8), which was added together with amidorphin-(8–26) to the enzymatic solution as nonamidated control peptide, was completely digested after 5 min. Results are mean ± SEM, $n = 4$.

postmortem degradation, we compared the ratio of amidorphin to amidorphin-(8–26) in various tissues at different times after death. Brains and pituitaries from 12 cows were put on ice within 30 min postmortem. Posterior pituitaries, hypothalami, and striata were dissected within 1 hr. One half of these tissues (i.e., pools of the respective areas from 6 cows) were immediately extracted, and the other half was extracted after 6 hr at room temperature. Amidorphin-immunoreactive peptides were analyzed by gel filtration and HPLC as described above. No change in the ratio of amidorphin to amidorphin-(8–26) between the different postmortem times could be observed in any of the three representative tissues investigated.

DISCUSSION

We have isolated and sequenced a C-terminally amidated peptide, amidorphin-(8–26), from bovine striatum. This nonopioid peptide can be considered a major product of the opioid peptide precursor proenkephalin in the brain, where it occurs in concentrations comparable to those of major opioid peptide products of this precursor, such as [Met]enkephalin, [Leu]enkephalin, and hepta- and octapeptide (15). The sequence of amidorphin-(8–26) corresponds to the C-terminal portion of the opioid peptide amidorphin, which we have

Table 3. Differential distribution of amidorphin and amidorphin-(8–26) in bovine tissues

Tissue	Total ir-AM, pmol/g tissue	AM, pmol/g tissue	AM-(8–26), pmol/g tissue	Ratio AM to AM-(8–26)
Adrenal medulla	1380	1380	<0.2	Only AM
Posterior pituitary	5210	680	3380	0.20
Hypothalamus	640	170	430	0.40
Midbrain	150	47	100	0.47
Brainstem	110	22	73	0.30
Striatum	440	<0.2	350	Only AM-(8–26)

The total immunoreactive amidorphin (ir-AM) was measured after gel filtration of pooled tissue extracts ($n = 8$). Authentic amidorphin (AM) and amidorphin-(8–26) [AM-(8–26)] were determined by HPLC (see Fig. 3); values have been corrected for recovery losses.

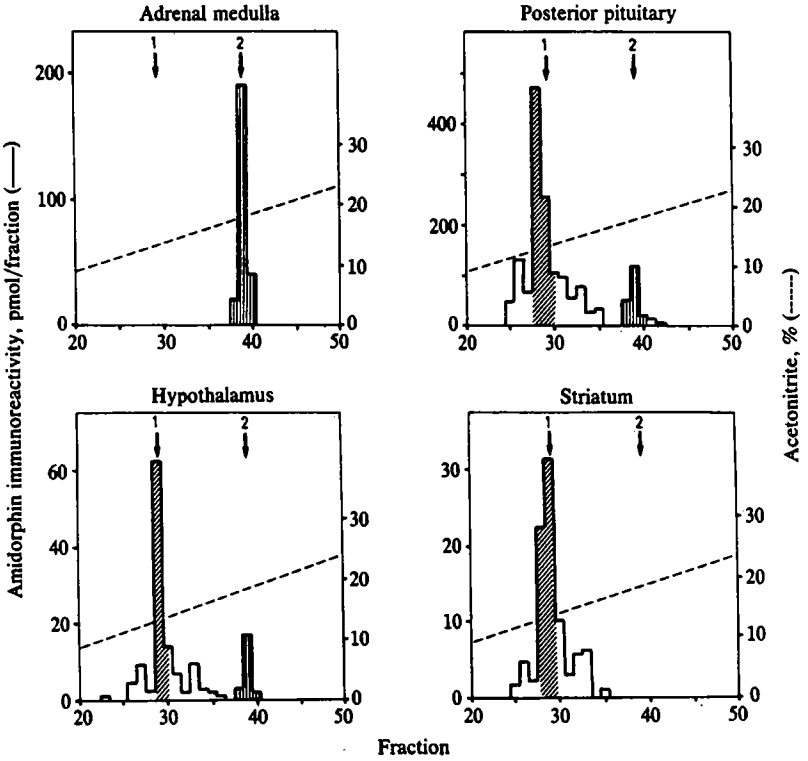

FIG. 3. HPLC profiles of immunoreactive amidorphin in four different bovine tissues. Amidorphin-immunoreactive peptides from the tissue extracts were purified by gel filtration, in which they eluted as a single peak in each case. The peak fractions were treated with H_2O_2 to oxidize amidorphin-related peptides to their respective methionine sulfoxide forms and passed through an Altex Ultrasphere ODS HPLC column (4.6 × 250 mm; particle size 5 μm); 2.0-ml fractions were collected and assayed for the amidorphin RIA. Arrow 1 indicates the elution position of oxidized amidorphin-(8–26), arrow 2 that of oxidized synthetic amidorphin.

recently isolated from bovine adrenal medulla (10). Amidorphin contains at its N terminus the opioid pentapeptide [Met]enkephalin, which is linked to the sequence of amidorphin-(8–26) by two basic lysine residues (see Fig. 4). Two basic residues are considered to be typical cleavage signals for the post-translational processing not only of opioid

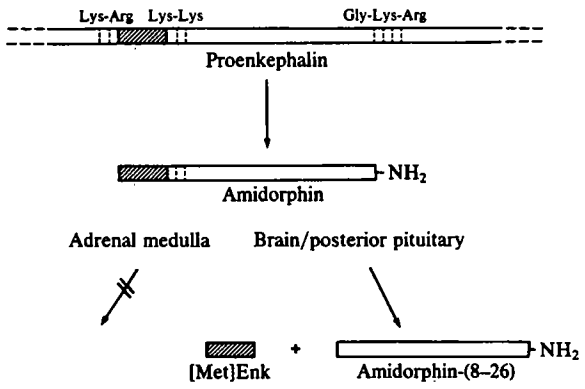

FIG. 4. Tissue-specific processing of bovine proenkephalin. In the adrenal medulla, proenkephalin seems to be processed only to amidorphin. Amidorphin, which corresponds to residues 104–129, may be liberated from proenkephalin by proteolytic processing at the flanking basic Lys-Arg residues and subsequent transformation of the C-terminal Gly residue into an amide group (10). However, in brain and posterior pituitary, as opposed to the adrenal medulla, amidorphin is obviously further processed at the Lys-Lys residues, giving rise to amidorphin-(8–26) and, possibly, [Met]enkephalin.

peptide precursors but also of protein/(neuro-)peptide precursors in general (16). Thus, in the brain, amidorphin obviously undergoes further proteolytic processing at these two lysine residues, giving rise to amidorphin-(8–26) and possibly [Met]enkephalin (Fig. 4). The proenkephalin system in the brain might, therefore, be an example of a precursor in which an opioid peptide ([Met]enkephalin) is linked with a nonopioid peptide [amidorphin-(8–26)] of possibly different biological function. Such a model is known from proopiomelanocortin, which contains the opioid peptide β-endorphin together with bioactive nonopioid peptides, such as corticotropin and α melanocyte-stimulating hormone (1). The physiological significance of amidorphin-(8–26) is not yet known. It is noteworthy, however, that this peptide contains a C-terminal amide group. This post-translational modification has been shown for several neuropeptides and hormones to be of essential importance for their biological activity—e.g., for corticotropin-releasing factor, gonadotropin-releasing factor, luteinizing hormone-releasing factor, α-melanocyte-stimulating hormone, cholecystokinin, and gastrin (17). For example, the biological activity of the hypothalamic peptide corticotropin-releasing factor is decreased by a factor of 1000 after removal of its C-terminal amide group (18).

In the adrenal medulla, where the opioid peptide amidorphin occurs in remarkably high concentrations and is a major product of proenkephalin, amidorphin-(8–26) was not detectable. Conversely, the brain predominantly or exclusively (striatum) contains high concentrations of amidorphin-(8–26) but only minor amounts of amidorphin. This is indicative of tissue-specific processing of proenkephalin. Thus, in contrast to the brain, proenkephalin seems to be

processed only to amidorphin in the adrenal medulla. In this tissue, the two lysine residues between the [Met]enkephalin- and amidorphin-(8–26) sequences of amidorphin obviously do not serve as processing signals as they do in the brain. This is of particular interest since other sequences with two basic residues, such as the Lys-Arg residues that precede the amidorphin sequence (residues 102–103) in bovine proenkephalin are indeed recognized as cleavage sites in the adrenal medulla and brain. These findings are consistent with the high specificity of those processing enzymes that are considered to cleave between two basic residues (19, 20). A similarly high degree of tissue specificity in post-translational processing events has been demonstrated in detail for proopiomelanocortin and prodynorphin (21–24). Although less information is so far available for proenkephalin, recent studies (10), including the present investigation, have begun to yield insights into the proteolytic processing of this precursor and may provide the basis for understanding the physiological significance of this system.

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